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TWO SPECIES OF THE *AMATHES C-NIGRUM* COMPLEX (LEPIDOPTERA: NOCTUIDAE) DISTINGUISHED BY ISOZYMES OF ADENYLATE KINASE AND BY SELECTED MORPHOLOGICAL CHARACTERS

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Abstract.—Two species of cutworms, *Xestia dolosa* Franclemont and *Xestia adela* Franclemont were separated electrophoretically by two different single-banded phenotypes of the enzyme adenylate kinase. Measurements of five morphological characters were compared by discriminant analysis, and the assignment of individuals, from collections containing both species, to two groups was in good agreement with that made by the enzyme. Subsequently three of the characters were selected which provide good separation, and a simplified discriminant process for practical application is discussed.

In 1971 pheromone tests carried out by Roelofs and Comeau suggested that males of a large form of *Amathes c-nigrum* (Linnaeus) were attracted to the cis isomer of 7-tetradecenyl acetate while males of a small form selected the trans isomer, but additional experiments using these compounds did not produce clear results. Recently Franclemont (1980) described the two forms of *A. c-nigrum* as two new species, basing his decision on differences in size, color, and genitalia morphology.

Xestia adela Franclemont, previously known as the small form of *A. c-nigrum* is widely distributed across North America with records extending from Alaska and the Northwest Territories to Virginia, Florida, Tennessee, Missouri, Kansas, Arizona, and Mexico (Crumb, 1927; McNay, 1959; Rings and Johnson, 1977). It is considered to be specifically distinct from the large form, now called *Xestia dolosa* Franclemont. *Xestia dolosa* occurs sym-

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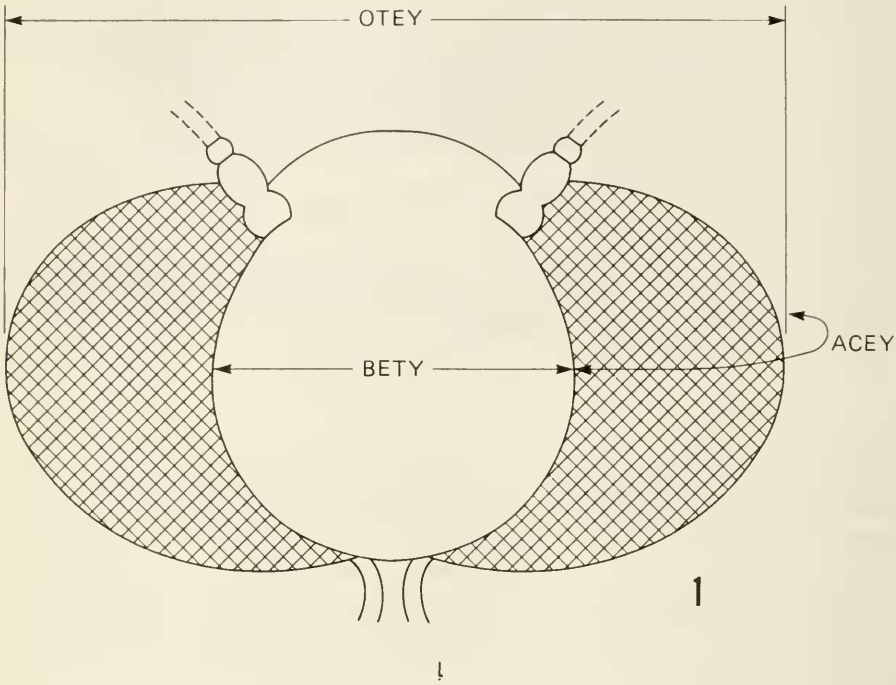

Fig. 1. Outline of front view of head of *Xestia adela* showing characters measured. See text for explanation of abbreviations.

patrically with *X. adela* in eastern North America and there is some evidence that the abundance of the larger species increases towards the southern limits of its range; Holland (1903), who reported *Noctua c-nigrum* as abundant throughout the Appalachian subregion, illustrated the species with an example which appears to be *X. dolosa*.

In sympatric populations it is often difficult to distinguish the two species using the rather subtle differences described by Franclemont (1980) and additional diagnostic characters are being sought.

In a current study of the electrophoretically detectable variation of a number of enzymes, originally undertaken to provide genetic information about the status of the two forms of *A. c-nigrum*, single pair matings were established from the progeny of local (Ottawa area) females of the small form, collected in early June, 1979. The enzyme adenylate kinase was found to occur as a single band (Adk^f) in these females and their progeny. In later collections (July, 1979) from the same location, some larger specimens were found which, when electrophoresed, showed a slower migrating band Adk^s;

Fig. 2. *Xestia adela* adult showing characters measured on fore wing.

their progeny also showed only the Adk^{s} band. Light trap collections received from London, Ontario (approximately 600 km from Ottawa and a somewhat different habitat) contained large and small specimens which also differentiated into Adk^{s} and Adk^{f} electromorphs. Three out of 159 individuals tested from both populations revealed a double-banded pattern Adk^{fs}.

These observations suggested that the Adk phenotypes of the moths might provide a useful diagnostic from which to investigate other characters capable of differentiating the two forms.

This paper reports the results of a discriminant analysis of selected morphological characters of individuals from field collections of different populations initially separated on the basis of their Adk phenotypes. The results are reported in terms of large (L) and small (S) segregates of the *Amathes c-nigrum* complex.

Materials and Methods

Moths.—The moths used in this study were obtained as adults from light traps set up at the following locations: (1) North Gower, Ontario (Ottawa area) and (2) London, Ontario (Talbotville and Hyde Park).

Insects obtained from the field were frozen at −80°C until used.

Eggs were obtained from 19 gravid females of *X. adela* and *X. dolosa* collected at North Gower in June, 1979, and the progeny reared to establish single pair matings. Adults of one line of *X. adela* with a fast migrating Adk band (Adk^{f}), and one line of *X. dolosa* with a slow migrating Adk band (Adk^{s}) were used as markers in electrophoretic runs.

The rearing techniques used are similar to those described by Hinks and Byers (1976) for species of *Euxoa*.

Preparation of moths.—The insects were thawed individually and one

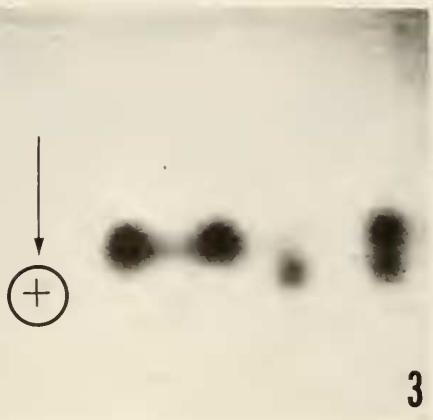

Fig. 3. Photograph of starch gel showing Adk isozymes of *Xestia dolosa* and *X. adela* (bands from left to right, 1 and 2 Adk^{s}, 3 Adk^{f}, 4 Adk^{fs}).

hind leg and the abdomen were removed and immediately refrozen. The remainder of the insect was pinned.

Extraction.—Hind legs were again thawed and then homogenized in 0.05 ml of distilled water in centrifuge tubes and centrifuged at 6000 *g* for 30 minutes.

Electrophoresis.—The techniques of horizontal starch gel electrophoresis and the staining method used for the enzyme adenylate kinase are similar to those described by Ayala et al. (1972). The gels contained 55 g electrostarch (Electrostarch Co., Madison, Wisconsin) in 440 ml of tris citric acid buffer at pH 7.1 and were run at 50 mA (110 v) for 4 hours. The stain buffer was slightly modified by adjusting to pH 8.0.

Morphology.—Measurements were obtained from the head (Fig. 1) and wings (Fig. 2) of each individual. Characters selected are as follows: (1) Across the widest point of the head (OTEY); (2) Maximum width of frons between the eyes (BETY); (3) Eye, posterior to anterior (ACEY); (4) Length

Table 1. Variances of five morphological characters of the *Amathes c-nigrum* complex, measurement error and natural variability.

	OTEY	BETY	ACEY	WIN 1	WIN 2
Measurement Variance × 12	0.06	0.18	0.10	1.28	1.63
Mean of all data (n = 159)	6.25	2.83	2.82	9.31	5.57
Within population variances × 157	13.96	5.40	4.18	86.44	130.98
Percent of within group variance attributable to measurement error	5.62	43.61	31.30	19.37	16.28

Table 2. Generalized squared distances among centroids of the forms and sexes of segregates (L and S) of the *Amathes c-nigrum* complex.

	L♂	L♀	S♂	S♀
L♂	—			
L♀	0.48	—		
S♂	11.69	8.49	—	
S♀	10.25	7.67	0.43	—

of the black area on the fore wing, from the anterior transverse line to the proximal margin of the reniform spot, measured along the cubital vein (WIN 1); and (5) Length of the orbicular spot (light triangular area within the black area) on the fore wing, between the two points terminating the slope of the triangle (WIN 2).

All were measured at a magnification of about 200 using a ×10 objective. Calculations were made from the actual unit measurements; each unit = 0.047 mm.

Numerical procedures.—The statistical methods used in this paper are given in many text books describing multivariate analysis (e.g. Anderson, 1958).

Homogeneity of covariance matrices was examined by Barlett's test, and the equality of mean vectors by Hotelling's T^2 test. The generalized squared distances are Mahalanobis based on the pooled covariance matrices and related to T^2 in the usual way.

Fisher's linear discriminant was found using the technique of Gower (1966).

All arithmetic was performed on an IBM 370/168 computer using Agriculture Canada's program S015, written by the Statistical Research Section of the Engineering and Statistical Research Institute.

Results

The adenylate kinase banding patterns of 159 field-collected male and female moths of the *A. c-nigrum* complex were recorded. Seventy-four in-

Table 3. Squared distances between spring and summer broods of L and S segregates of the *Amathes c-nigrum* complex at 2 locations in Ontario.

Population	1	2	3	4
1. N. Gower Spring L				
2. London Spring L	3.28			
3. N. Gower Summer S	21.28	26.32		
4. N. Gower Spring S	8.19	15.16	5.81	
5. London Spring S	15.08	12.43	9.75	8.75

Table 4. Mean vectors, discriminant axes and functions, and relative importance of 5 and 3 variables of L and S segregates of the *Amathes c-nigrum* complex.

Variables	Means		Overall Means	Ranking based on Forwards Step-wise Procedure	Ranking based on F-ratios in a One Way Analysis of Variance	Discriminant			Discriminant		
							Functions			Functions	
	L	S				Axis	L	S	Axis	L	S
OTEY	6.66	5.89	6.25	1	1	4.71	72.69	56.56	4.39	86.70	71.94
BETY	2.93	2.74	2.83	4	3	−1.62	7.77	13.31			
ACEY	2.97	2.68	2.82	5	2	0.40	30.25	28.88			
WIN 1	9.50	9.14	9.31	3	4	−0.54	−2.02	−0.17	−0.61	−1.56	0.49
WIN 2	5.39	5.73	5.57	2	5	−0.55	−6.53	−4.63	−0.55	−6.32	−4.48
Sample size	74	85	159	–	–	Const.	−271.0	−209.5	Const.	−264.25	−201.85

Table 5. Within population sums of squares (on diagonal), cross products (below diagonal) and correlations (above diagonal) matrix (df = 157).

Variables	OTEY	BETY	ACEY	WIN 1	WIN 2
OTEY	13.96	0.652	0.113	0.654	0.475
BETY	5.66	5.40	0.562	0.505	0.307
ACEY	5.45	2.67	4.18	0.491	0.383
WIN 1	22.72	10.91	9.33	86.44	0.508
WIN 2	20.30	8.16	8.96	54.01	130.98

dividuals having the slower migrating band (Adk^s) were considered as large (L); 85 individuals which had the faster migrating band (Adk^f) were classified as small (S). There were also 3 double-banded individuals which are denoted by D (Fig. 3), and which therefore cannot be assigned unequivocally.

Before attempting to obtain a linear discriminant function based on the five measurements described above, some preliminary studies were undertaken:

(1) To estimate a measure of the reliability of the measurements, 12 specimens were measured on two separate occasions. The expected value of the differences between the paired measurements is zero; their estimated variances are given in Table 1. The first variable is thus very reliable with only 5.62% of within group variance being attributable to measurement error. The other variables are much less reliable, probably because of the lack of precision in their demarcation.

(2) To investigate possible sexual dimorphism, all the specimens were divided into four groups, L males and females, and S males and females. The generalized squared distances between pairs of centroids (i.e. the pooled squared differences in the overall means between populations) of the four groups are given in Table 2. Because the values of the squared distances between the sexes within a group are not significant by the T^2 test (they are less than 6% of those between L and S forms), it is concluded that for these

Table 6. Assignments obtained using discriminants based on 5 and 3 variables of L and S segregates of the *Amathes c-nigrum* complex.

		Assigned Membership (5 variables)		Assigned Membership (3 variables)	
		L	S	L	S
Known membership	L	73	1	73	1
	S	3	82	5	80
	D	1	2	2	1

Table 7. Squared distances between the 6 misclassified individuals and the centroids of L and S segregates of the *Amathes c-nigrum* complex using 3 variables.

Specimen Classified Electrophoretically as	L	S
L	6.35	0.68
S	2.61	2.98
S	2.58	3.02
S	1.96	3.78
S	1.77	4.06
S	2.56	3.03

five measurements no sexual dimorphism is shown; sex differences are subsequently ignored.

(3) To investigate within group seasonal dimorphism the specimens were separated into spring and summer broods and locations. Five populations were designated as follows (Table 3): spring and summer broods of North Gower S group (3 and 4); spring brood of North Gower L group (1); and spring broods of London L and S groups (2 and 5). The squared distances between all five populations are shown in Table 3 (the covariance matrix excluded laboratory reared specimens and unclassified individuals). Between populations 3 and 4 the value obtained is 5.81, but when these two populations are considered alone it is reduced to 4.40. Between populations 1 and 4 the value is 8.19, but when these two are considered alone it is increased to 11.5. The analytical tests applied indicate that there is a difference in size between the spring and summer broods from North Gower which is smaller than the difference between the two forms occurring there. The tests also indicate that there may be a difference between similar populations from different locations (e.g. 4 and 5) which is of the same order as that between 3 and 4.

A number of preliminary tests of hypotheses were carried out in order to determine the value of obtaining a linear discriminant function.

(1) The test for equality of covariance matrices gave an x^2 of 43.6 as an estimate of χ^2_{15}; since the 5% critical value for this is 25.0, the hypothesis that there is no difference between the L and S forms seems unlikely.

(2) Using the multivariate Behrens-Fisher test of equality of mean vectors given unequal covariance matrices, Hotelling's multivariate T^2 test gave an F-ratio of 90.6 for no difference in the mean vectors with 5 and 153 degrees of freedom which is also highly significant.

The conclusion from these is that there is good evidence that not only are the mean vectors different, but also that the relationships among the variables is not the same in the two groups, and so there is something to discriminate. Table 4 gives the mean vectors, the discriminant axes, the discriminant functions obtained, and also the rankings of the variables based

Table 8. Squared distances between the three D specimens and the centroids of the L and S segregates of the *Amathes c-nigrum* complex using 5 and 3 variables respectively.

Specimen	5 variables		3 variables	
	L	S	L	S
D1	10.82	0.01	8.22	0.23
D2	3.30	2.60	1.77	4.05
D3	0.04	12.21	0.01	11.36

on a forwards stepwise estimate of their discriminating power. Table 5 gives the estimated within population sums of squares on the diagonal, the cross products among the five variables below the diagonal, and correlations above; the correlations among the variables are all positive and significantly different from zero.

The within population variance along the discriminant axis is estimated at 2.96. It is worth noting that L is larger than S in all characters but the second wing measurement, and so the differences between them are not only in size. Using the first set of discriminant functions (columns 7 and 8, Table 4) the assignment of the 159 individuals and the three D individuals is as in Table 6 (columns 1 and 2).

Because of the relative unreliability of two of the head measurements (Table 1) and also because they are the least important in the stepwise procedure the analyses were repeated using just OTEY, WIN 1, and WIN 2. Since neither the covariance matrices based on these 3 variables are equal ($x^2 = 19.34$, $\chi_6^2 = 12.59$), nor are the means equal ($F^3_{155} = 145$), the population differences shown by the five variables persist with the three (Table 4). The axes (column 9), not unexpectedly, are slightly different from those in the first part of the table, but are obviously related. The within population variance on this discriminant axis is 2.82 which is very slightly less than the value for all five variables, and indicates that discarding two variables has had only a small impact. In confirmation, since the generalized squared distance was reduced from 11.75 to 11.18 which is not significant by the Behrens-Fisher multivariate T^2 test, it is concluded that nothing is gained by retaining the "noisy" variables.

In Table 6 (columns 3 and 4) the assignments using the second set of discriminants, of Table 4 are given.

Several differences between the assignments using five and then three variables are apparent; two more S individuals are assigned to the L group, giving a total of six individuals misclassified, and one member of the D group is switched from S to L. It is of interest to give the generalized squared distances between the six misclassified individuals and the centroids of the L and S groups (Table 7).

The distances between the three D specimens and the L and S centroids

are shown in Table 8. Specimen D2 transfers from S to L when only three characters are utilized; D1 remains closest to S and D3 closest to L.

Twenty-nine specimens from across N. America were taken from the Canadian National Collection and measured. Using the second set of discriminants in Table 4 assignments were made to L or S groups. One individual from Mexico and one from North Dakota were assigned to group L which was unexpected, because all the western material previously examined has been considered as the small form (*X. adela* Franclemont, 1980).

Discussion

Taxonomic information obtained from enzyme variation is now commonplace in studies of populations and species complexes, and an enzyme locus which provides significant differentiation between two populations can be employed as a diagnostic character (Ayala and Powell, 1972).

The enzyme adenylate kinase, used here to differentiate two new species of *Xestia* from Ontario, is concerned with transphosphorylation and energy transfer and catalyzes the reaction 2 ADP $\rightleftharpoons$ ATP + AMP (ADP and hexokinase were used in our reaction mixture). In man Adk exhibits soluble and mitochondrial isozymes determined by separate structural gene loci. The soluble form predominates in adult cardiac muscle (Edwards and Hopkinson, 1977). Examples of allele frequencies of soluble Adk in adult Lepidoptera are given for *Euphydryas editha* (Boisduval) and *E. chalcedona* (Doubleday and Hewitson) (Nymphalidae) by McKechnie et al. (1975) and for *Aricia artaxerxes* (Fabricius) (Lycaenidae) by Jelnes (1975). In both species of nymphalid butterflies the same Adk allele predominated in all populations sampled, while other alleles at this locus occurred at very low frequencies, so that the enzyme is not diagnostic in this case. In the lycaenid only one population of the three sampled was polymorphic for Adk, the other two were monomorphic for the same allele. In this case only one homozygote and the heterozygote were distinctive.

In our samples of *Xestia* two different alleles distinguished two species in all but three individuals. The occurrence of two bands in three out of 159 samples must remain unexplained until breeding experiments (currently in progress) have determined if hybridization can occur between the two species in the laboratory, and if known hybrids portray both parent bands.

Discriminant analysis of morphological measurements resulted in good agreement with the enzyme segregation with less than 4% of the individuals misclassified. It is possible that the discriminants used here may not be reliable for populations distant from Ontario; nevertheless, using them, the assignment of 29 individuals obtained from across N. America seems reasonable. The two exceptions warrant further study of the distribution of *X. dolosa*.

These results provide additional diagnostic characters and give strong support to the recognition by Franclemont (1980) of two new species within North American populations of the spotted cutworm.

Since potential users of the results of this paper may be unfamiliar with discriminant analysis, an example of its application for identification purposes may be helpful. In the following, an unidentified specimen (U) is measured and found to have: OTEY = 5.3, WIN 1 = 8.6 and WIN 2 = 5.2. Using the discriminants shown in the last two columns of Table 4: Score for U as *Xestia dolosa* = (86.70 × 5.3) − (1.56 × 8.6) − (6.32 × 5.2) − 264.25 = 148.98; Score for U as *Xestia adela* = (71.94 × 5.3) + (0.49 × 8.6) − (4.48 × 5.2) − 201.85 = 160.35.

Since the computed value for *X. adela* is larger, the conclusion is that U belongs to that taxon. If the two values had been approximately equal a decision could not be made.

Acknowledgments

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